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Dissociation of the Spectrin-Ankyrin Complex as a Basis for the Loss of Na,K-ATPase Polarity Following Ischemic Injury in MDCK Cells

Submitted by

Robert Woroniecki, MD¹, Jean R. Ferdinand, MS¹,
Jon S. Morrow, MD, PhD², and Prasad Devarajan, MD^{1,3}

¹Pediatric Nephrology, Albert Einstein College of Medicine, New York,

²Pathology, Yale University School of Medicine, New Haven, and

³Nephrology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

*Correspondence to P. Devarajan, Nephrology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, MLC 7022, Cincinnati, OH 45229-3039. Phone: (513) 636-4531. FAX: (53) 636-7407. E-mail: prasad.devarajan@chmcc.org

Abbreviated title: Loss of Na,K-ATPase polarity following ischemia

Abstract

The polarized distribution of Na,K-ATPase at the basolateral membranes of renal tubule epithelial cells is maintained via a tethering interaction with the underlying spectrin-ankyrin cytoskeleton. In this study, we have explored the mechanism underlying the loss of Na,K-ATPase polarity following ischemic injury in MDCK cells, utilizing a novel antibody raised against a recently-described kidney-specific isoform of ankyrin. In control MDCK cells, ankyrin was co-localized with Na,K-ATPase at the basolateral membrane. ATP depletion resulted in a duration-dependent mislocation of Na,K-ATPase and ankyrin throughout the cytoplasm. Co-localization studies showed partial overlap between the distribution of ankyrin and Na,K-ATPase at all periods following ATP depletion. By immunoprecipitation with anti-ankyrin antibody, the mislocated Na,K-ATPase remained bound to ankyrin at all time points following ATP depletion. However, the interaction between ankyrin and spectrin was markedly diminished within 3 h of ATP depletion, and was completely lost after 6 h. In solution binding assays using a fusion peptide of GST with the ankyrin binding domain of Na,K-ATPase, a complex with ankyrin was detected at all time points following ATP depletion, but spectrin was lost from the complex in a duration-dependent manner. The loss of spectrin binding was not attributable to spectrin degradation, but was associated with hyperphosphorylation of ankyrin. The results suggest that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface may contribute to the loss of Na,K-ATPase polarity following ischemic injury, and reaffirm a critical adapter role for ankyrin in the normal maintenance of Na,K-ATPase polarity.

Key words: ankyrin, Na,K-ATPase, spectrin, polarity, ischemia, phosphorylation.

Introduction

Tethering interactions between integral membrane proteins and the underlying spectrin-based cytoskeleton play key roles in several cellular activities, including the establishment and maintenance of ordered membrane domains (1). Ankyrins are a family of conserved proteins that have emerged as adapter molecules mediating such linkages, since they possess binding sites for a variety of integral membrane proteins as well as for spectrin (1-6). A particularly well characterized example is the linkage between ankyrin and α -Na,K-ATPase, mediated primarily by residues within ankyrin's repeats domain and the second cytoplasmic domain of α -Na,K-ATPase (7-9). This interaction is especially critical to the cells lining the kidney tubules, which vectorially transport ions and nutrients via mechanisms that are dependent on the polarized basolateral co-localization of Na,K-ATPase, ankyrin and spectrin (10-12). One of the major consequences of acute ischemic injury to renal tubule cells is the disruption of polarity, with a co-ordinate mislocation of Na,K-ATPase, ankyrin and spectrin to alternate cellular sites (12-14). This has been demonstrated both *in vitro* (15-17) and in human biopsy samples (18), with important implications for the abnormal handling of sodium and glucose by the postischemic kidney (19-21). However, the molecular basis for the loss of Na,K-ATPase polarity after ischemic renal injury remains incompletely understood.

We have recently cloned and characterized a novel renal isoform of ankyrin, termed AnkG190 based on the predicted size of the polypeptide (9). Others and we have shown that AnkG190 is the major isoform associated with the basolateral domain of renal

epithelial cells (9, 22). In this study, we have developed an anti-peptide polyclonal antibody directed to the unique N-terminal sequences of AnkG190. We demonstrate that AnkG190 interacts with α -Na,K-ATPase at the lateral domain of Madin Darby canine kidney (MDCK) cells. ATP depletion results in a duration-dependent loss of α -Na,K-ATPase polarity. Using a series of complementary assays, we show that ankyrin remains bound to α -Na,K-ATPase following ATP depletion, but that the interaction between ankyrin and spectrin is lost. We suggest that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface, possibly mediated by a post-translational mechanism involving serine/threonine and tyrosine phosphorylation of ankyrin, may contribute to the loss of Na,K-ATPase polarity that follows ischemic injury.

Materials and Methods

Cell culture and ATP depletion:

MDCK type II cells, an established polarized renal tubule epithelial cell line obtained from American Type Culture Collection (Rockville, MD), were passaged in complete DMEM with 10% fetal bovine serum (Gibco, Gaithersburg, MD) and analyzed within 1 day of reaching confluence. Cells were grown on six-well tissue culture-treated polystyrene plates (Costar, Cambridge, MA) except for microscopy, for which they were grown on cover slips placed within the wells. In a separate set of experiments, cells were grown on Transwell filters (Costar) to ensure complete polarization. ATP depletion was used as a well-established model of reversible ischemic injury to MDCK cells (17, 23-26). Briefly, confluent cells were washed with PBS and incubated for varying time periods in glucose-free DMEM (Gibco) in the presence of 1 μ M antimycin A (Sigma, St. Louis, MO) as an inhibitor of oxidative phosphorylation (26). We have previously shown that MDCK cells subjected to this protocol undergo partial but reversible ATP depletion (26). After about 8 h, a subset of cells so stressed initiate the process of apoptosis, but the majority remain adherent and viable (26).

Preparation of polyclonal antibodies to AnkG190:

Affinity-purified polyclonal antibodies were generated (Quality Controlled Biochemicals, Hopkinton, MA) in two rabbits following injection of a glutaraldehyde-conjugated synthetic peptide containing residues 15-31 of AnkG190. These residues were chosen

since they are unique to AnkG190 (9). Antibody titers of hyperimmune sera were monitored by ELISA using the BSA-coupled peptide.

Microscopy:

Immunofluorescence microscopy was performed at room temperature as previously described (9, 10, 27, 28). Briefly, 4-micron sections of paraffin-embedded rat kidney, or MDCK cells at confluence or following varying periods of ATP depletion, were fixed with acetone for 15 min, blocked in goat serum for 30 min, incubated in primary antibody in 2% BSA containing 10% goat serum for 60 min, washed, incubated in secondary antibodies conjugated to Cy2 or Cy3 (Amersham, Arlington Heights, IL), and visualized with a microscope (Olympus AX70, Lake Success, NY) equipped for epilumination. The antibodies used were the polyclonal to AnkG190 at 1:200 dilution and a monoclonal to α -Na,K-ATPase at 1:1000 (Upstate Biotechnology, Lake Placid, NY).

Immunoprecipitations:

Immunoprecipitations were performed as previously described (9, 28). Briefly, cells in six well plates were lysed for 20 min at 4 °C in 2 ml of IP buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.5% deoxycholate, 1% Nonidet P-40, 1X Complete[®] protease inhibitor from Roche Applied Science, Indianapolis, IN). In a separate set of experiments, cells were extracted *in situ* with a low salt buffer (10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 0.5% Triton X-100, 1X Complete[®] protease inhibitor) in order to isolate a cytosolic fraction, as previously described (7-9, 27, 28). The lysates were centrifuged for 1 min at 10,000 X g, and precleared by a 60-

min incubation at 4 °C with 25 µl preimmune serum and 200 µl of a 50% protein A-Sepharose solution (Upstate Biotechnology). The cleared supernate was incubated with 20 µl of the polyclonal AnkG190 antibody for 4 h at 4 °C, and for an additional 2 h with 200 µl of a 50% protein A-Sepharose solution. The lysates were centrifuged for 1 min at 10,000 X g, washed three times with IP buffer, and the pellet subjected to SDS-PAGE and Western analysis. The antibodies used were the polyclonal to AnkG190 at 1:200 dilution, a monoclonal to α -Na,K-ATPase at 1:5000 (Upstate Biotechnology), a polyclonal to β II spectrin (10D) at 1:200 dilution (9), and a monoclonal to tubulin at 1:10,000 (Sigma).

Ankyrin binding assay:

Solution binding assays were performed using glutathione *S*-transferase (GST) fusion peptides as previously described (7, 8). The minimal ankyrin binding (MAB) domain of α -Na,K-ATPase lies within residues 142-166, and the preparation and purification of recombinant peptides representing this region have been previously detailed (7, 8). A construct encoding for MAB was expressed in bacteria as a GST fusion using the pGEX prokaryotic expression system (Pharmacia, Piscataway, NJ), and purified using glutathione-agarose (7, 8). GST alone was expressed as a control peptide. Proteins were analyzed by SDS-PAGE followed by staining with Coomassie Blue, and were quantified by the Bradford assay (Bio-Rad, Hercules, CA). Each fusion protein (GST or GST-MAB, 50 µg at 1 mg/ml) was conjugated to 50 µl of a 50% slurry of glutathione-agarose for 1 h at 4 °C with gentle rotation, and incubated at 4 °C overnight with 1 ml of a cytoskeletal fraction (300 µg of total protein) from confluent MDCK cells extracted *in*

situ using a buffer containing 10 mM Pipes, 500 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100, and 1X Complete (Roche) protease inhibitors (7, 8). The beads were pelleted, washed twice with PBS, and aliquots analyzed by SDS-PAGE followed by Western blotting and enhanced chemiluminescence (Amersham) with antibodies as above.

Ankyrin phosphorylation assays:

The phosphorylation status of ankyrin was examined using protocols as recommended by the manufacturer (Upstate Biotechnology). Briefly, cells were preincubated for 60 min with protease inhibitor (1X Complete[®] protease inhibitor from Roche) and a cocktail of phosphatase inhibitors including 1 mM sodium vanadate (Calbiochem), 10 mM okadaic acid (Gibco) and 1 nM calyculin A (Gibco). Cells were then extracted *in situ* with high salt lysis buffer (500 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0), and lysates centrifuges at 14,000 rpm for 10 min at 4°C. The clear supernates (1 ml) were incubated overnight at 4°C with either 20 µl of preconjugated antiphosphotyrosine agarose (Upstate Biotechnology), or with a combination of 20 µl of antiphosphoserine/threonine antibody and 20 µl of IgG agarose (Upstate Biotechnology). Bound complexes were recovered by centrifugation, washed with 1X PBS, and subjected to Western analysis with antibody to AnkG190 as described above.

Results

Ankyrin is polarized to the basolateral membrane of kidney tubules and MDCK cells:

We generated polyclonal antibodies directed to the unique N-terminal sequences of AnkG190. The antibody specifically recognized an immunoreactive peptide at 190 kDa in rat kidney lysates, and a 210 kDa peptide in MDCK cell lysates, whereas the pre-immune serum was devoid of cross-reactivity (Figure 1). By immunofluorescence microscopy, AnkG190 was localized predominantly to the basolateral domains of kidney tubule cells (not shown) and cultured MDCK cells (Figure 1), while no staining was observed with the pre-immune serum (not shown). Double-staining of MDCK cells revealed a co-localization of AnkG190 with α -Na,K-ATPase along the basolateral membrane (Figure 2, Panel G).

ATP depletion results in a duration-dependent loss of polarized distribution of Na,K-ATPase and ankyrin:

The well-established ATP depletion model was used to study the behavior of ankyrin and Na,K-ATPase following ischemic injury in MDCK cells. Within 3 h of ATP depletion, there was a partial loss of basolateral membrane staining of AnkG190 (Figure 2B) and α -Na,K-ATPase (Figure 2E), with both proteins beginning to assume a punctate cytoplasmic distribution characteristic of internalized Na,K-ATPase. By 6 h of ATP depletion, the cytoplasmic mislocation of both AnkG190 (Figure 2C) and α -Na,K-ATPase (Figure 2F) was complete, and no staining of either protein was visible at the basolateral membrane. Another uniform observation was that the abundance of

immunoreactive ankyrin was increased after 6 h of ATP depletion (2C vs 2A). This is consistent with our previous findings of enhanced ankyrin expression in a rat model of renal ischemia (29).

Ankyrin remains bound to Na,K-ATPase following ATP depletion:

Since a tethering interaction with ankyrin is crucial to the normal basolateral distribution of Na,K-ATPase, it was of interest to determine the behavior of ankyrin in cells with mislocated Na,K-ATPase following ATP depletion. Double immunostaining revealed that AnkG190 remained partially co-localized with α -Na,K-ATPase at all time periods following ATP depletion (Figure 2H and 2I).

The finding that ankyrin continues to interact with Na,K-ATPase following ATP depletion was confirmed using two additional complementary assays. First, analysis of the polyclonal AnkG190 antibody immunoprecipitates demonstrated that control cells contained functional complexes of AnkG190, α -Na,K-ATPase, and β II-spectrin (Figure 3A). Conversely, in cells depleted of ATP, α -Na,K-ATPase continued to immunoprecipitate with ankyrin, whereas spectrin was lost from the complex. The loss of spectrin from the complex was duration-dependent (Figure 3A), and could not be attributed to its degradation, since the 240 kDa immunoreactive spectrin band remained intact in whole cell lysates at all time periods examined (Figure 3B). In a separate set of experiments, with cells grown on Transwell filters to optimize the generation and maintenance of cell polarity, identical results were obtained (not shown). In another set of experiments, the cells were extracted with a low salt buffer to isolate a purely cytosolic

fraction. Ankyrin in this cytosolic fraction continued to associate with α -Na,K-ATPase after 6 h of ATP depletion, but not with spectrin (not shown).

In the second assay, the *in vitro* interaction of ankyrin from ATP-depleted cell lysates with a GST fusion protein containing the minimal ankyrin binding domain of α -Na,K-ATPase (GST-MAB) was evaluated. In control cells, this peptide bound strongly to an ankyrin-spectrin complex (Figure 4). Following ATP depletion, GST-MAB continues to avidly bind ankyrin, but spectrin was progressively lost from the complex as a function of the duration of ATP depletion (Figure 4). The negative control, GST alone, did not interact with ankyrin or spectrin.

An additional observation gleaned from Figures 3 and 4 pertains to the enhanced ankyrin expression following ATP depletion. A significant increase in the abundance of immunoreactive ankyrin was noted following 6-12 h of ATP depletion, consistent with the enhanced ankyrin immunofluorescence observed in Figure 2 and with our previous *in vivo* observations (29). Densitometric analysis of multiple blots revealed that ankyrin protein abundance increased by 2.5 ± 0.5 fold (mean \pm SD) at 6 and 12 h of ATP depletion.

Ankyrin undergoes phosphorylation following ATP depletion:

Since membrane-cytoskeletal interactions in general, and the interactions between spectrin and ankyrin in particular, are often regulated by phosphorylation (1, 30, 31), it was of interest to determine whether the phosphorylation status of ankyrin changed following ATP depletion. This was measured by evaluating the amount of ankyrin that

was present in anti-phosphotyrosine or anti-phosphoserine/threonine precipitates. Surprisingly, despite the diminished ATP content of these cells, increases in both tyrosine and serine/threonine phosphorylation on ankyrin were detected, as shown in Figure 5. Enhanced phosphorylation was detected as early as 3 h and persisted through 6 h of ATP depletion. These changes correlated with the rate of spectrin loss from the ankyrin-Na,K-ATPase complex.

Discussion

We have developed a polyclonal antibody directed to the unique N-terminal sequences of AnkG190 and demonstrated that AnkG190 interacts with α -Na,K-ATPase at the lateral domain of polarized MDCK cells. Upon ATP depletion, there is a duration-dependent loss of Na,K-ATPase and ankyrin polarity. Using a series of complementary assays, we have demonstrated that ankyrin remains bound to Na,K-ATPase following ATP depletion, but that the interaction between ankyrin and spectrin is lost. We suggest that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface may contribute to the loss of Na,K-ATPase polarity and contribute to its internalization following ATP depletion.

Ischemic renal injury remains a leading cause of acute renal failure, and despite important technical advances in treatment the associated mortality and morbidity remain dismally high (12-14). An improved understanding of the pathophysiology from a cellular and molecular standpoint may facilitate the development of novel therapeutic interventions. Although the pathogenesis of acute renal failure is clearly multi-factorial, persistent afferent arteriolar vasoconstriction is considered an important factor, and has been postulated to occur secondary to the loss of proximal tubule cell polarity (12). Previous studies have established that ischemic injury modeled by ATP depletion results in a disruption of polarized basolateral membrane distribution of Na,K-ATPase, ankyrin and spectrin in cultured proximal tubule cells (15-17), leading to impaired sodium reabsorption (19, 20). Loss of Na,K-ATPase polarity and increased delivery of filtered

sodium to the macula densa have also been documented in human cadaveric kidneys following ischemia-reperfusion injury (18, 21). It is reasonable to hypothesize that the ensuing activation of tubuloglomerular feedback contributes to the persistent vasoconstriction characteristic of ischemic acute renal failure. However, the molecular basis for the loss of Na,K-ATPase polarity after ischemic renal injury remains incompletely understood.

Several lines of evidence gleaned from the present study suggest that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface may be responsible for the loss of Na,K-ATPase polarity following ATP depletion. First, the internalized and mislocalized α -Na,K-ATPase remains substantially co-localized with ankyrin. Second, ankyrin remains complexed with α -Na,K-ATPase in immunoprecipitates, whereas spectrin is lost from the complex in proportion to the duration of ATP depletion. Third, the isolated ankyrin binding domain of Na,K-ATPase continues to interact *in vitro* with ankyrin after ATP depletion whereas spectrin does not. Fourth, β II spectrin remains intact and does not undergo degradation following ATP depletion. Collectively, these results support earlier studies demonstrating the lack of association between ankyrin and spectrin following ATP depletion in LLC-PK1 cells (17), and demonstrate the persistence of a crucial ankyrin-Na,K-ATPase linkage. Others have similarly shown that in large measure, both spectrin and ankyrin remain intact following ischemic injury to MDCK cells (30).

The phosphorylation state of ankyrin is an important determinant of its interaction with spectrin (31-36). Unphosphorylated ankyrin preferentially binds to the fully functional tetrameric spectrin unit rather than to dimers with a 10-fold greater affinity; this preferential binding is abolished by phosphorylation. Although the sites and physiological significance of phosphorylation remain unclear, an attractive mechanism by which ATP depletion might influence spectrin-ankyrin interactions would be by altering the phosphorylation status of ankyrin. Our findings indicate that ATP depletion results paradoxically in a marked increase in both tyrosine and serine/threonine phosphorylation of ankyrin, suggesting a possible biochemical mechanism underlying the loss of association between spectrin and ankyrin. While the role of phosphorylation in ankyrin is unknown, it has been shown that hyperphosphorylation of ankyrin on serine/threonine in avian red blood cells by casein kinase II (CKII) suppresses its ability to bind spectrin (35, 36). Casein kinase II is also known to phosphorylate spectrin (37). Normally, ankyrin and spectrin undergo a futile cycle of rapid phosphorylation and dephosphorylation, reflecting dynamic control of membrane and cytoskeletal organization. During a period of ATP depletion, this cycle is presumably interrupted or unbalanced. The mechanisms that account for changes in kinase/phosphatase activity under these conditions remain unexplored. Another factor that may influence the spectrin-ankyrin interaction following ATP depletion includes changes in the oligomeric status of spectrin *per se*. In erythrocytes, the affinity of spectrin for ankyrin is cooperatively linked to its oligomeric state, with ankyrin favoring spectrin oligomers and tetramers over spectrin dimers (32, 34). Since spectrin's oligomerization state can be influenced by a variety of factors including phosphorylation, Ca^{++} , calmodulin, and proteolysis (33, 38), these factors may

also contribute under conditions of renal tubule cell injury to the deconstruction of the cortical spectrin-ankyrin skeleton.

Finally, an additional observation offered by this study is the finding that immunodetectable ankyrin is upregulated following ATP depletion in MDCK cells, consistent with our previous findings in a rat model of renal ischemia (29). Although the molecular mechanisms and significance of this change are unclear, it is intriguing to speculate that the overexpressed ankyrin, *via* its continued ability to interact with Na,K-ATPase, may play a role in the restoration of cell polarity during recovery from ischemia, lending emerging evidence for the notion that ankyrin-spectrin binding is a necessary cofactor for the appropriate membrane delivery and sorting of a subset of proteins that includes Na,K-ATPase (28), anion exchanger (39), CD45 (40), and others (41).

In summary, the present study suggests that a dissociation of the membrane-cytoskeleton complex at the spectrin-ankyrin interface may contribute to the loss of Na,K-ATPase surface display and polarity following ATP depletion. It will be important in future studies to examine the phosphorylation status of ankyrin following ischemic injury in animal models, to further elucidate the possible mechanisms underlying the selective loss of spectrin binding and preferential retention of the interaction between ankyrin and Na,K-ATPase.

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Figure Legends

Figure 1: Ankyrin is polarized to the basolateral membrane of MDCK cells: Left panel: Western blot of MDCK cells or rat kidney lysates with pre-immune serum (Pre) or polyclonal AnkG190 antibody (Ank). Molecular weight markers are shown along the left margin. Right panel: Immunofluorescence microscopy of MDCK cells with AnkG190 antibody. Bar, 10 microns. Results were reproducible in three separate experiments.

Figure 2: ATP depletion results in a duration-dependent loss of polarized distribution of Na,K-ATPase and ankyrin: MDCK cells, untreated (left panels), or ATP depleted for 3 h (middle panels) or 6 h (right panels) were double stained with antibodies to ankyrin (red, A-C) and α -Na,K-ATPase (green, D-F). The bottom panels (G-H) represent merged images. Bar, 10 microns. Results are representative of three experiments.

Figure 3: Ankyrin remains bound to Na,K-ATPase following ATP depletion - immunoprecipitation assays: Panel A: MDCK cells, untreated (Con) or ATP depleted for various periods as shown, were analyzed for complexes with ankyrin. The negative control was beads alone (Beads). Equal aliquots of cell lysates were probed with anti-tubulin antibody prior to precipitation to verify equal loading of samples. Panel B: Western blot of cell lysates at various time periods as indicated, with anti-spectrin antibody. The numbers on the left depict molecular weights in kDa. The figure is representative of three experiments.

Figure 4: Ankyrin remains bound to Na,K-ATPase following ATP depletion - solution binding assays: Top panel: Coomassie Blue stained gel of the recombinant peptides GST alone (negative control) and GST-MAB. Middle and bottom panels: Western blots with antibodies to ankyrin and spectrin respectively. The numbers on the left depict molecular weights in kDa. The figure is representative of two experiments.

Figure 5: Ankyrin is phosphorylated following ATP depletion: Western blot with ankyrin antibody of MDCK cell lysates following various periods of ATP depletion and immunoprecipitation with anti-phosphotyrosine or anti-phosphoserine/threonine. Equal aliquots of cell lysates were probed with anti-tubulin antibody prior to precipitation to verify equal loading of samples.

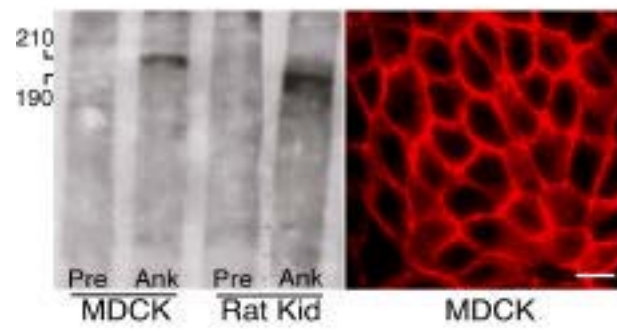
Figure 1

Figure 2

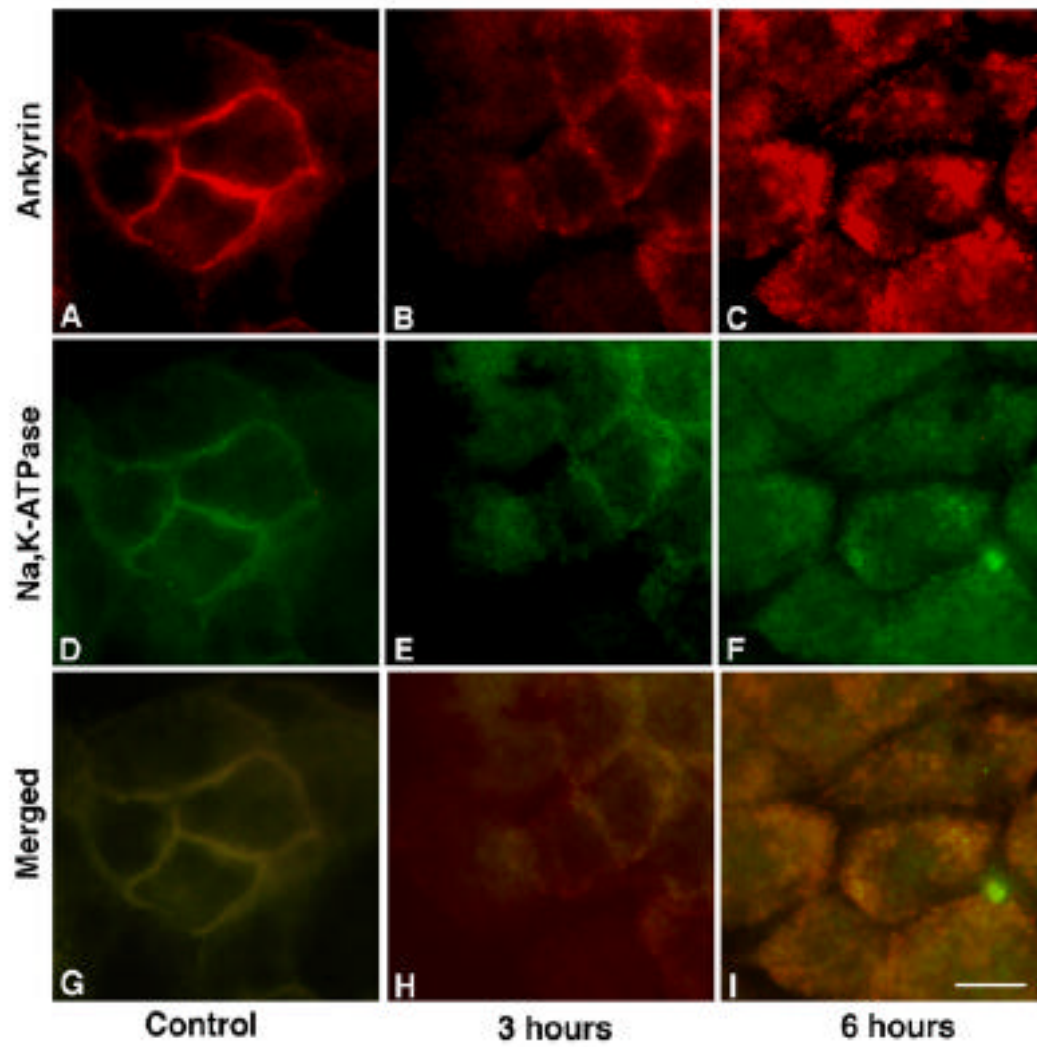


Figure 3

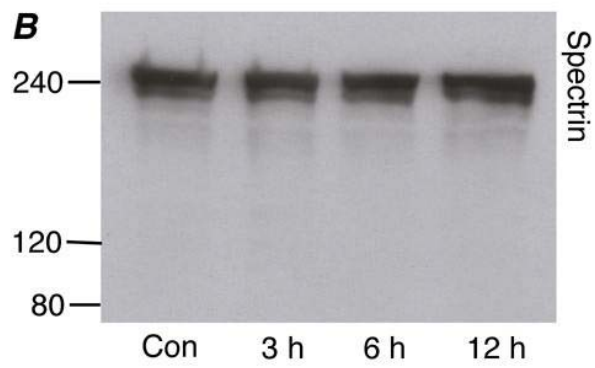
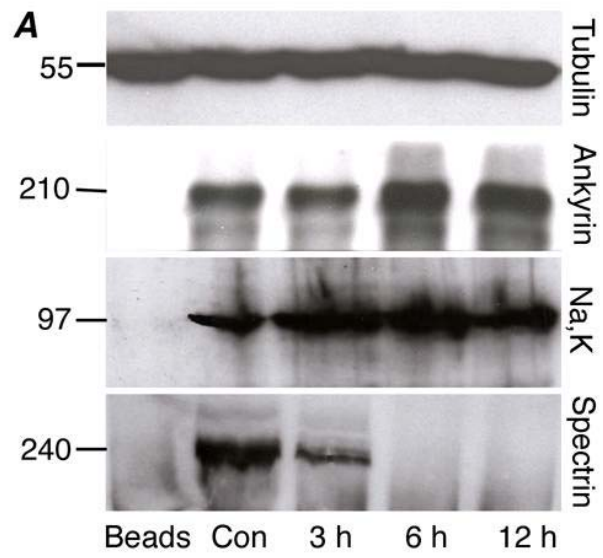


Figure 4

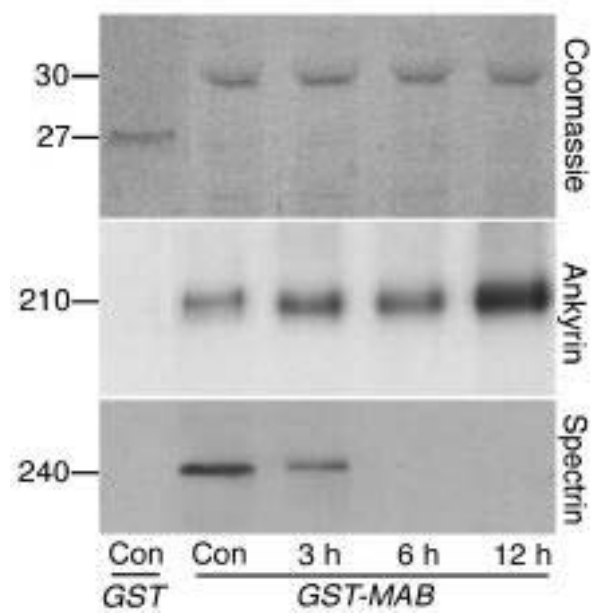


Figure 5

